Studies on the Biosynthesis of Pyocyanine. (III) : On the Effect of Iron and Other Heavy Metals

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Studies on the Biosynthesis of Pyocyanine. (III)

On the Effect of Iron and Other Heavy Metals

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In the present work, it has been found that when pepton solution or its hydrolysate was treated with active charcoal, their increasing effect on pyocyanine formation disappeared, and that this reaction of charcoal was due to adsorption of Fe which should be regarded as one of the effective constituents of peptone.

Using the bacterial strain of *Pseudomonas aeruginosa* capable of pigmentation in the synthetic medium mentioned in the previous paper, the effects of Fe and other heavy metal ions were examined on pyocyanine formation and bacterial growth. Among various heavy metals, Cu also exhibited the effect similar to that of Fe on pigmentation. On the other hand bacterial growth and pyocyanine formation were observed even in the medium containing KCN, although the period of lag phases was prolonged.

INTRODUCTION

As was already mentioned in the previous paper,[1] peptone had been regarded as the material essential for pyocyanine formation.

Similar effect was also expected with acid-hydrolysate of peptone. However, it has been found that their remarkable effect on pyocyanine formation disappeared when peptone or its hydrolysate was treated with active charcoal, whereas the bacterial growth was not so affected by charcoal treatment. When peptone was incinerated to ashes, it was no longer expected to be available for pyocyanine formation, so that the effective constituent was relating to organic substance. However, the effect of peptone lost by charcoal treatment was found to be recovered by the addition of $FeSO_4$, so that one of the factors necessary for pigmentation must be due to Fe. On the other hand, it was found that pyocyanine formation could, in some cases, take place even in the synthetic medium without peptone, according to the kind of bacterial strain. A decreasing effect of charcoal treatment of peptone solution on pyocyanine formation was observed to be more remarkable with increasing amount of active charcoal, even when suitable amount of Fe was added to the medium, hence other promoting factor for pigmentation was regarded to be eliminated from peptone together with Fe by charcoal treatment, although the bacterial growth was fairly satisfactory. In the present paper, the view of the effect of Fe and other heavy metal ions on pigmentation will be presented prior to other effective constituents of peptone.

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EXPERIMENTAL AND DISCUSSION

Effect of Peptone treated by Charcoal Adsorption

As was already mentioned, if peptone or its hydrolysate was treated with active charcoal, their increasing effect on pyocyanine formation should be decreased whatever the corresponding amount of Fe lost by charcoal treatment might be supplied. Fig. 1 represents the effect of adsorption treatment of peptone with active charcoal on pyocyanine formation and the recovery of pigmentation by supplement of Fe.

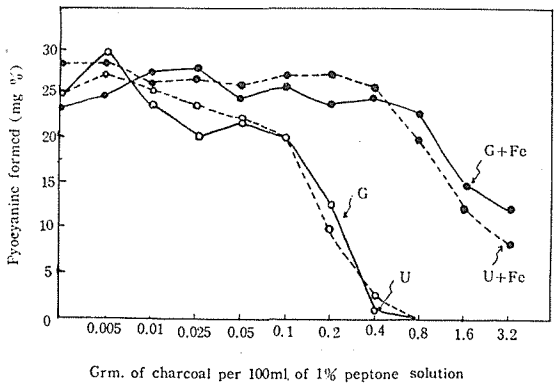


Fig. 1. Effect of charcoal treatment of peptone on pyocyanine formation. G, medium was composed of 3% glycerol, 1% peptone, 0.1% glutamic acid, 0.1% NH_4NO_3, 0.025% $MgSO_47H_2O$ and 0.025% K_2HPO_4. U, glutamic acid and NH_4NO_3 in G were replaced by 0.2% urea. G+Fe, U+Fe; 0.0005% $FeSO_47H_2O$ was added to each medium, respectively.

It will be seen in the figure that pyocyanine formation was remarkably decreased as an increasing amount of active charcoal was used for the treatment, and that by the addition of Fe, pyocyanine formation was almost completely recovered. And in the case in which excessive amounts (more than 0.8 g per 100ml of 1 % peptone solution) of active charcoal were employed, the recovery was not satisfactory even by the addition of Fe. In this case, it was noted that according to the increasing amount of active charcoal, formation of green fluorescent pigment was noticeably increased with decreasing effect on pyocyanine formation. In the medium containing peptone, the addition of Fe compound is of no significance, because Fe is usually recognized in peptone solution.

In order to know the amount of Fe in peptone, the following experiment was carried out : 1 g of peptone was incinerated to ashes, dissolved in HCl and diluted with water to 100 ml. This solution was used for Fe estimation by the colorimetric method[2] and it was found that the amount of Fe was corresponding to 0.6 to 0.9 mg of $Fe_2(SO_4)_3$ per g of peptone in every case of commercial preparations. No pigmentation is not attributable to the absence of Fe but to its insufficiency, becuse appreciable amount of Fe which sustains the bacterial growth, is always recognized in the residual part of peptone treated by charcoal adsorption. From the experiment mentioned above, it may be concluded that the effective

constituent of peptone does not reveal a controlling effect but does a promoting effect on pyocyanine formation.

Therefore, peptone is not necessarily an indispensable substance for pigmentation, at least from the ordinary viewpoint of the strain of *Pseudomonas aeruginosa*, so that pyocyanine formation occurs in some cases even in the synthetic medium without peptone, according to the kind of bacterial strain.

Table 1. Effect of Fe on pyocyanine formation.

Fe(m*M*)		1.0	0.5	0.25	0.10	0.05	0.025	0.010	0.005	0.0025	0.0010	0.0005
$FeSO_4$	B_1	—	0.2	0.3	0.3	0.3	0.5	0.6	0.5	0.3	—	—
	B_2	—	trace	0.2	0.4	0.6	1.2	1.1	0.8	0.5	—	—
$Fe_2(SO_4)_3$	B_1	—	—	—	0.3	0.3	0.5	0.4	0.3	0.2	0.1	trace
	B_2	—	—	trace	0.2	0.6	1.2	1.2	0.6	0.2	trace	—
$K_4Fe(CN)_6$	B_1	0.7	0.6	*	0.4	0.2	0.4	0.4	0.2	trace	—	—
	B_2	1.1	1.5	0.8	0.9	*	0.8	0.8	0.6	0.8	—	—
$K_3Fe(CN)_6$	B_1	0.6	*	0.4	0.4	0.3	0.6	0.2	trace	—	—	—
	B_2	0.9	0.8	0.8	1.0	0.9	0.6	0.6	0.1	—	—	—
$Fe_2(SO_4)_3$ +Tartrate	B_1	—	0.4	0.4	0.6	0.2	0.6	0.3	0.4	trace	—	—
	B_2	0.6	1.8	1.4	0.9	0.9	0.5	1.5	0.5	0.3	0.1	—

Medium : 2% glycerol, 0.5% glutamic acid, 0.1% NH_4NO_3, 0.05% $MgSO_47H_2O$, 0.025% K_2HPO_4 and requisite amount of Fe.

Incubation was performed at 37° for 4 days. Data show the amount of pyocyanine expressed in % $\times 10^{-3}$. B_1, B_2 : kinds of bacterial strain. — Non-pigmentation, * no measurement.

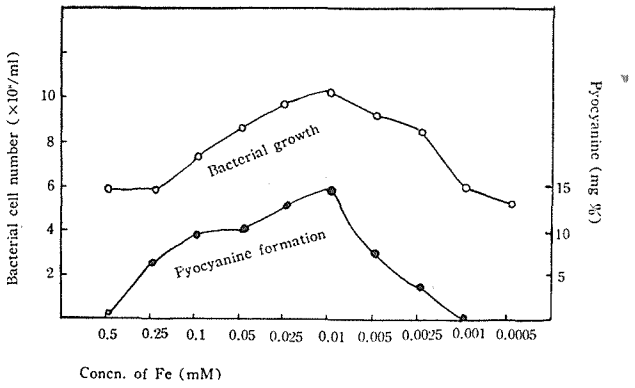


Fig. 2. Effect of Fe on pyocyanine formation. Medium : 2% glycerol, 0.5% glutamic acid, 0.1% NH_4NO_3, 0.05% $MgSO_47H_2O$, 0.025% K_2HPO_4 and requisite concentration of $FeSO_47H_2O$.
Incubation was carried out at 37° for 4 days.

Effect of Concetration of Fe

Tables 1, 2 and 3 show the results of the experiments on the effect of Fe. There exists a noticeable discrepancy between the limiting concentrations of Fe for pyocyanine formation and for bacterial growth. It is clearly seen in Fig. 2 that excessive part of Fe used for bacterial growth will be offered for pyocyanine

formation. Any significant difference was not pointed out between the valencies of Fe ion, *i. e.* ferric and ferrous or ferri- and ferrocyanides, but the requisite amount of these ion compounds differed according to the kinds of bacterial strain or of Fe compounds. In general, the optimum concentration of Fe for pyocyanine formation was observed to be in the range from 10^{-4} to 10^{-5} *M*, and at the concentration higher than 0.25m *M* where Fe was almost precipitated as its hydroxide, a decreasing effect was observed. However it should be noted that increasing effect on pyocyanine formation was observed with such a concentration of ferri- or ferrocyanide as $10^{-1}M$ where any precipitation was not revealed (Table 1). Waring *et al.*[3] have shown that the precipitated Fe apparently adsorbed on bacterial cell surface would be injurious for bacterial growth, whereas high concentration of Fe was not toxic as long as it was all in solution. The mechanism of the inhibitory action of Fe in high concentration on pyocyanine formation may also be based on the above fact, since the same results as with ferricyanide were obtained with ferric sulfate when tartrate was added in order to prevent ion precipitation (Table 1).

Effect of Fe on Bacterial Growth

In order to test the effect of Fe on bacterial growth, the following experiments were performed. It was a difficult matter to eliminate Fe completely from culture medium, and yet a satisfactory Fe-deficient medium was prepared by modifying the method of Poppenheimer[4] by means of calcium phosphate gel.

By using tartrate instead of phosphate, results of the experiments were found to be more satisfactory. Bacterial growth was represented by turbidity which was standardized by number of bacterial colonies as will be illustrated in the following procedure : the dilution of the cultured solution incubated at 37° for 24 hours was made with distilled water, in such ways as one, at first, in a hundred and then one successively in ten, so that 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} dilutions were arranged. With each dilution plate culture on nutrient

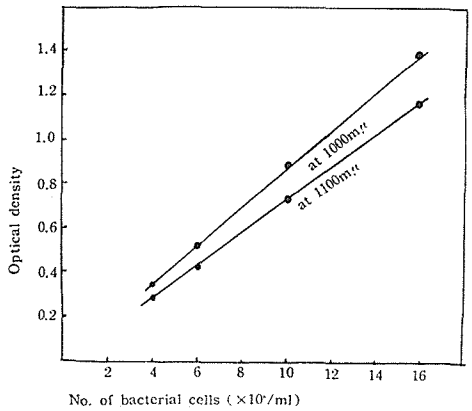


Fig. 3. Relation between turbidity and bacterial cell number. Turbidity was expressed by optical density at 1000 and 1100 mμ.

agar, incubations were carried out in the ordinary way at 37° for 24 hours, and the original cell number of cultured solution was calculated from the arithmetic mean values of the the number of colonies in each plate.

The standard curve expressing the relation between the cell number and the turbidities of each dilution which were measured using Beckman spectrophotometer, model DU, at 1000 or 1100mμ in order to avoid the influence of pyocyanine or of other products, was found to be given as a straight line (Fig. 3). It was

Table 2. Effect of Fe on bacterial growth.

$FeSO_4 7H_2O$ (%)		0.01	0.005	0.001	0.0005	0.00025	0.0001	0.00005	Nil
Bacterial cell number	B_1	5.8	8.4	10.8	11.2	9.8	6.8	3.8	1.8
($\times 10^8$/ml)	B_2	5.6	9.6	11.4	12.0	9.6	6.6	3.8	2.4

B_1 and B_2 are kinds of bacterial strain.
Basal medium : 2% glycerol, 0.2% urea, 0.05% $MgSO_4 7H_2O$ and 0.025% K_2HPO_4. Incubation was performed at 37° for 24 hours.

indicated from Table 2 that even with the medium expected to be free from Fe, bacterial growth was considerably revealed. In spite of the further experiments of the successive bacterial cultures, such a conclusion has not decidedly been obtained as the bacterial strain used in the present experiments could grow on the medium lacking Fe, because Fe-porphyrin enzyme such as catalase was observed with the bacterial cells. As had been pointed out by Mueller,[5] any effort might not be successful in preparing the Fe-less medium, and yet it will be possible to conclude the necessity of Fe for bacterial growth.

According to the information of Waring[6], *Pseudomonas aeruginosa* requires three or four times more Fe than other aerobic bacteria probably because of its higher cytochrome content. Furthermore, the following technique was attempted: after removing the bacterial cells grown on Fe-deficient medium at 37° for 48 hours, the cultured solution made clear was divided into two culture flasks, and to one of the flasks Fe was added. When these culture flasks were pasteurized, inoculated and incubated under the ordinary condition, the bacterial growth was, to some extent, observed in the presence of Fe whereas the growth was scarcely seen in the absence of Fe. From these results, it may be concluded that Fe is indispensable for both pyocyanine formation and bacterial growth.

It is of interest to note that a remarkable effect of Fe-cyanide was observed not only on pyocyanine formation but also on bacterial growth. Although Fe has been observed to be an essential factor for bacterial growth, the effect on the growth of the Fe-deficient medium was much inferior to its effect on pyocyanine formation. In other words, it was not unusual to point out the case in which bacterial cell multiplication attained to a considerably satisfactory state, but any pyocyanine formation did not take place; and the other case in which there observed a considerably poor growth of bacteria, but, appreciable pyocyanine formation (Table 3). Therefore, two limiting concentrations of Fe should be considered for pyocyanine formation and for bacterial growth probably according to their enzyme

systems in which requisite amounts of Fe are independently different. However, the mechanism of the effect of Fe remains obscure whether it shows an activity on pigmentation by incorporating into the enzyme structure or as a cofactor in enzyme systeme of pyocyanine formation.

Table 3. Effect of Fe on pyocyanine formation.

	Pyocyanine formed (%)	Growth
No.1	0.002	0.446
No.2	—	0.520

No. 1: medium; 2% glycerol, 0.1% NH_4NO_3, 0.025% $MgSO_4 7H_2O$, 0.025% K_2HPO_4 and 0.0005% $FeSO_4 7H_2O$.
No. 2: medium; 2% glycerol, 0.2% urea, 0.05% $MgSO_4 7H_2O$, 0.025% K_2HPO_4 and 1% peptone treated by charcoal.
Bacterial growth was expressed by turbidity. — Non-pigmentation.

As an attempt, the following experiment was carried out: to the cultured solution with the medium containing such a small amount of Fe as the becterial growth was fairly satisfactory while pyocyanine formation was not observed, 0.0005% $Fe_2(SO_4)_3$ was added at the stage of 48 hours' incubation when the bacterial growth curve was attained to be stationary phase. The result is shown in Fig. 4. By the addition of Fe pyocyanine formation was, for the first time,

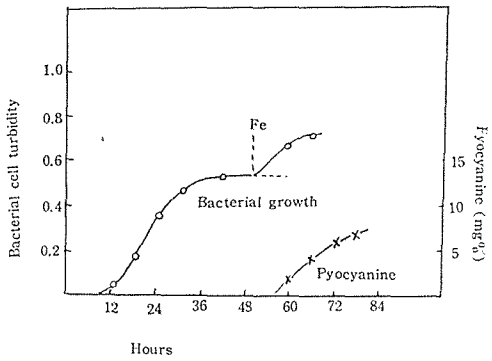


Fig. 4. Effect of Fe on pyocyanine formation. Medium: 2% glycerol, 0.2% urea, 0.025% $MgSO_4 7H_2O$, and 1% peptone treated by active charcoal. 0.0005% $Fe_2(SO_4)_3$ was added after 48 hours' incubation.

revealed, but the effect of Fe on pigmentation was hardly recognized after so long period. On the other hand, bacterial growth was accelerated soon after the addition of Fe. Hence, it is assumed that Fe would serve rather as the constituent of enzyme structure than as the cofactor of the enzyme action in pyocyanine synthesis system. At any rate, the enzyme system in pyocyanine synthesis should be regarded to be independent of that in bacterial growth.

Effect of Other Heavy Metals

The experiments with other metals by which Fe could be substituted were carried out and the result shown in Table 4 was obtained. Cu was found to be the only one metal ion which served to some extent as a substitute for Fe, while other metal ions such as Zn, Co, Ni, Cd, Pb and Mn did not reveal any effect on pyocyanine formation. On the bacterial growth, Zn ion produced, more or less, the promotion; Co, Ni and Cd revealed an inhibitory action, while any effect was not observed with Pb.

Table 4. Effect of various heavy metals on pyocyanine formation.

Metals (%)	0.05	0.025	0.010	0.005	0.001	0.0005	0.0001
$FeSO_4 7H_2O$	+	+	+	+	+	+	trace
$CuSo_4 5H_2O$	—	—	trace	+	+	+	—
$ZnSO_4 2H_2O$	—	—	—	—	—	—	—
$CoCl_2 6H_2O$	*	*	*	*	—	—	—
$NiSO_4 6H_2O$	*	*	*	—	—	—	—
$CdSO_4 4H_2O$	*	*	*	—	—	—	—
$PbSO_4$	—	—	—	—	—	—	—
$MnSO_4 H_2O$	*	*	—	—	—	—	—
Controll	—	—	—	—	—	—	—

Basal medium was the same as in Table 2. + Pigmentation positive, — non-pigmentation, * no multiplication.

Effect of Cu

Since the optimum concentration of Cu was considered to be at a considerably high level, 0.2% Na-tartrate was added to the medium in order to avoid the precipitation of Cu-hydroxide, and the concentration of $CuSO_4$ was adjusted colorimetrically prior to the preparation of the medium. As shown in Table 5, Cu ion showing the effect on pyocyanine formation also revealed to some extent a promoting effect on bacterial growth, but at the concentration higher than 0.05% of $CuSO_4 5H_2O$ no pigmentation was pointed out, especially when the medium contained no glutamic acid by which the bacterial growth would be protected

Table 5. Effect of Cu on pyocyanine formation.

$CuSO_4 5H_2O$ (%)		0.05	0.025	0.010	0.005	0.0025	0.0005	0.0001	Nil
Pyocyanine (%)	B_1	—	trace	0.001	0.002	0.002	0.001	trace	—
growth	B_1	0.320	0.422	0.532	0.524	0.534	0.438	0.380	0.328
Pyocyanine (%)	B_2	trace	trace	0.001	0.003	0.002	0.001	trace	—
growth	B_2	0.310	0.440	0.538	0.542	0.538	0.510	0.440	0.380

B_1, B_2: kinds of bacterial strain.
Basal medium: 2% glycerol, 0.5% glutamic acid, 0.1% NH_4NO_3, 0.05% $MgSO_4 7H_2O$ and 0.025% K_2HPO_4.
Bacteril growth was expressed by turbidity.

from inhibitory action of Cu ion. Although Cu is far inferior to Fe in effect on pigmentation, it is of interest to point out that such metal ions as reveal an activity on oxidation-reduction system in organisms are effective for pyocyanine formation.

Effect of HCN

In connection with the effect of Fe, influence of cyanide on pyocyanine formation or bacterial growth was tested. The preparation of the medium were performed as follows : as KCN is unstable in solutional state, its solution was added lastly to the medium after pasteurization, pH was adjusted to 7.8 and incubated at 30° so as to avoid its evaporation. The results are shown in Fig. 5.

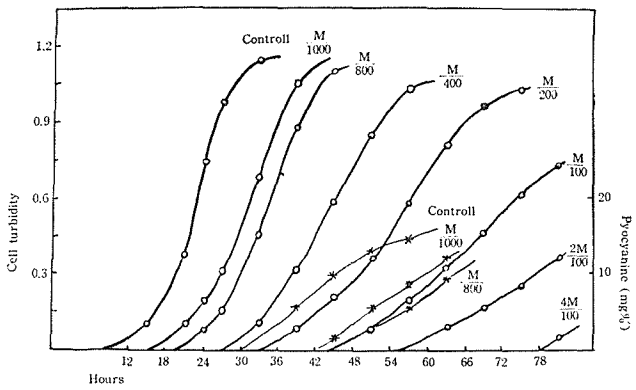
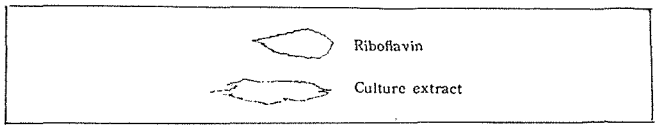


Fig. 5. Effect of KCN. Medium : 2% glycerol, 1% peptone, 0.2% urea, 0.025% $MgSO_47H_2O$ and 0.025% K_2HPO_4. KCN was added in the requisite amount from 0.001 to 0.04*M*. (○-○-○) Bacterial growth, (*-*-*) pyocyanine formation.

It was possible to observe both bacterial growth and pyocyanine formation with the medium containing KCN, although the lag phases in both cases were prolonged, especially with the synthetic medium without peptone or at higher concentration of cyanide. Johnson *et al.*[7,8,9] have reported that some strains of *Pseudomonas aeruginosa* possessed the ability to form hydrocyanic acid. Although the formation of hydrocyanide could not be demonstrated with the present strain, but it was found that bacterial growth in the medium containing cyanide was based on the capacity of the bacteria to break down cyanide, because in the stage where bacterial growth and pyocyanine formation were sufficiently performed, the existence of cyanide was hardly recognized.

However, in the case in which cyanide was used at high concentration, a part of the cyanides supplied was observed to remain unchanged even in the stage mentioned above. Accordingly, it is considered that pyocyanine may be formed in the presence of appreciable amount of cyanide without depending on the decomposition of it.

In these cases, a small amount of fluorescent yellow pigment considered to be a flavin derivative was detected from butanol extract of cultured solution by paper



→ n-butanol-acetic acid-water (5 : 1 : 2).
Fig. 6. Paper chromatography of culture extract.

chromatography (Fig. 6). It is assumed that the present strain can grow on the medium containing cyanide owing to the capacity of decomposing it or to the existence of specific respiration system relating to the bacterial growth.

SUMMARY

1. It was found that when peptone solution or its hydrolysate was treated with active carbon, their increasing effects on pyocyanine formation disappeared and that this reaction of active carbon was ascribable to the adsorption of Fe which was one of the effective constituents of peptone. The amount of Fe existing in peptone was estimated to be corresponding to 0.6 to 0.9 mg of $Fe_2(SO_4)_3$ per g of peptone in major case of commercial preparations.

2. It was ascertained that Fe was an essential factor for pyocyanine formation and for bacterial growth, and that the limiting concentration of Fe for both cases are different from each other : for the former, 10^{-4} to $10^{-5}M$, and for the latter, $5\times10^{-7}M$.

3. As regards the form of Fe, not only ferrous or ferric compound but also ferro- or ferricyanide are useful for pyocyanine formation as well as bacterial growth.

4. Cu was found to be a substitute, to some extent, for Fe not only for pyocyanine formation but also for bacterial growth.

5. It was found that with the medium containing KCN, bacterial growth and pyocyanine formation were performed, although the lag phases in both systems were prolonged, and that in this case, a small amount of fluorescent yellow pigment considered as flavin derivative was detected.

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